Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

A preliminary neutron Laue diffraction study of the aspartic proteinase endothiapepsin

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Received 15 April 1999
Accepted 10 January 2000

Until now, no aspartic proteinase has been subjected to a successful neutron diffraction analysis, owing to the limited size of the crystals. However, the recent development of the neutron Laue technique at ILL and EMBL (Grenoble) has allowed the collection of data to 2.2 Å on a complex of endothiapepsin with a transition-state analogue. The objective is to define the positions of the protons at the active site by refinement using the neutron data. In line with work on serine proteinases, where neutron diffraction has provided some of the most definitive data on the catalytic mechanism, it is expected that this work will have a major significance for studies of the aspartic proteinase enzymes.

1. Introduction

Aspartic proteinases are a family of enzymes involved in a number of important physiological and pathological processes. Examples include renin, which is involved in elevating blood pressure *via* the renin–angiotension system (Dhanaraj *et al.*, 1992), and HIV proteinase, which is involved in retroviral maturation (Wlodawer *et al.*, 1989). They are also implicated in peptic ulcer disease (pepsin) and tumour invasiveness (cathepsins D and E; Baldwin *et al.*, 1993). Inhibitors of renin were developed extensively as potential anti-hypertensive agents (see Dhanaraj & Cooper, 1997) and inhibitors of the HIV proteinase have been shown to have therapeutic action in the treatment of AIDS (Roberts *et al.*, 1990).

Aspartic proteinases possess two catalytic aspartate residues (32 and 215 in porcine pepsin numbering) whose side chains are held in close proximity by an intricate arrangement of hydrogen bonds (Davies, 1990; Bailey & Cooper, 1994). A solvent molecule bound tightly to both aspartate carboxyl groups is presumed to take part in the catalytic mechanism (Pearl & Blundell, 1984). Current proposals for the mechanism are largely based on X-ray inhibitor structures (*e.g.* Suguna *et al.*, 1987; Veerapandian *et al.*, 1992), but differ in the assignment of protonation states to the catalytic groups during the reaction. Since the active-site H atoms cannot be located by current X-ray analyses, their putative positions have so far been inferred from the local geometry of surrounding polar atoms.

Neutron protein crystallography can offer a powerful adjunct to X-ray analysis at the resolutions typical of X-ray protein structure determinations (d > 1.5 Å) by enabling key details of the hydrogen structure to be revealed (Schoenborn, 1994). H and/or D atoms can be more readily located in a corresponding neutron analysis because the scattering amplitudes of H/D for neutrons are closely similar to those of other biological atoms.

The use of neutron diffraction in understanding enzyme mechanisms was pioneered by Kossiakoff & Spencer (1980), whose analysis of trypsin contributed significantly to current understanding of serine-proteinase catalysis. Similar work on lysozyme (Mason *et al.*, 1984) and ribonuclease (Wlodawer *et al.*, 1983) has established the protonation states of the catalytic groups, with important implications for their mechanisms of action.

The monochromatic neutron fluxes used for these analyses were relatively weak. Hence, these experiments required large sample volumes (>5 mm^3) and protracted time scales (weeks or months). This handicap can be overcome in part by using a Laue-diffraction geometry (Wilkinson & Lehmann, 1991). This provides a more rapid and efficient survey of reciprocal space, maximizing the flux at the sample and stimulating large numbers of reflections simultaneously over all incident wavelengths. A neutron Laue diffractometer (LADI) designed to exploit this advantage for protein crystallography is now installed on cold guide H142 at ILL. The detector consists of a large Gd_2O_3-doped neutron-sensitive image plate (400 × 800 mm) mounted on a cylindrical camera (318 mm diameter) that completely encircles the sample (Cipriani *et al.*, 1996). Narrow (quasi-Laue) bandpasses (λ = 3.0, 3.5, 6.0 Å; $d\lambda/\lambda$ = 8–25%) can be selected using Ni/Ti multilayer filters (Hoghoj *et al.*, 1996) in order to reduce the reflection overlap and

Table 1
Data-processing statistics for the neutron Laue data set collected from the endothiapepsin–H261 complex to 2.2 Å resolution.

d_{min} (Å)	R_{merge}	R_{cum}	$I/\sigma(I)$	Completeness (%)	Multiplicity
6.58	0.048	0.048	13.0	79.2	1.8
5.25	0.063	0.058	10.0	86.7	2.0
4.50	0.060	0.059	10.2	90.9	2.1
4.00	0.070	0.063	8.5	90.6	2.2
3.64	0.080	0.068	7.5	93.2	2.3
3.36	0.102	0.074	5.9	90.3	2.2
3.13	0.129	0.081	4.9	87.7	2.2
2.95	0.136	0.087	4.6	84.4	2.1
2.80	0.164	0.091	4.1	78.4	2.0
2.66	0.209	0.097	3.1	70.6	1.8
2.55	0.242	0.105	2.7	72.2	1.8
2.44	0.239	0.112	2.6	75.3	1.9
2.35	0.260	0.121	2.3	80.5	1.9
2.27	0.250	0.129	2.5	79.3	2.0
2.20	0.261	0.137	2.5	78.0	2.1
Total	0.137	0.137	4.5	80.4	2.0

incoherent scattering background from biological crystals with large unit cells. As a further consequence, almost all reflections (>99%) recorded on the LADI detector are singlets and thus are amenable to standard crystallographic analysis. The instrument is optimized for study of protein systems at medium resolution (>1.5 Å), sufficient to locate individual H atoms of special interest, water structures or other small molecules that can be labelled with deuterium to make them particularly visible. Studies of lysozyme using this instrument (Niimura *et al.*, 1997; Bon *et al.*, 1999) have provided much data on proton locations at the catalytic centre and the structure and dynamics of the surrounding solvent. The order of magnitude gains in data-collection rates that have been achieved make feasible studies of larger protein systems than have previously been possible (Helliwell, 1997).

We have performed preliminary neutron Laue studies of the fungal aspartic proteinase endothiapepsin (M_W = 33 kDa), with the ultimate objective of locating the active-site H atoms in complexes with transition-state analogues. Neutron data have been collected from a complex of endothiapepsin and the inhibitor H261 using the LADI instrument at ILL (Grenoble). These data extend to a resolution of 2.2 Å. Since the inhibitor possesses an isostere which mimics the putative transition state (Bailey & Cooper, 1994), we expect that the interactions made by the inhibitor will be similar to those formed transiently by the tetrahedral intermediate during catalysis. Thus, locating crucial protons at the active site will provide important information on the mechanism of action. This work also represents the largest macromolecular neutron structure to be analysed to date.

2. Methods

The co-crystals of endothiapepsin complexed with H261 were obtained by an adaptation of the method of Moews & Bunn (1970). This involved adding a tenfold molar excess of inhibitor to enzyme at a concentration of 2.0 mg ml^{-1} in 100 m*M* sodium acetate buffer at pH 4.5. Finely ground ammonium sulfate was added until slight turbidity became apparent (around 55% saturation), at which point the solution was Millipore filtered and, if necessary, a few drops of acetone were added to clear any remaining turbidity. Crystals appeared after several weeks; the crystal used for neutron diffraction was stored in mother liquor allowing it to grow for a total of 11 years.

A crystal of the endothiapepsin–H261 complex having dimensions of 1.8 × 1.4 × 1.4 mm was selected for data collection. In order to reduce the contribution of the large incoherent neutron-scattering cross section of hydrogen to the experimental background, the solvent and labile H atoms of the protein were exchanged by vapour-diffusion equilibration against deuterated mother liquor. This was achieved by mounting the crystal in a capillary and then equilibrating against several changes of a 90% deuterated mother-liquor solution and finally against a 95% deuterated solution. Neutron data to 2.2 Å were collected from this co-crystal at room temperature in a three-week experiment at ILL as detailed below.

3. Data collection and reduction

Quasi-Laue neutron data were collected on LADI installed on end-station LADI/T17 of cold neutron guide H142 at ILL (Myles *et al.*, 1998). A Ni/Ti multilayer wavelength selector was used to select a dλ/λ 20% bandpass centered at 3.20 Å. The spectral range was determined by time-of-flight techniques.

Data extending to 2.2 Å resolution were collected from the H261 complex at two crystal settings. A total of 14 data frames were collected with a spindle rotation of 8° between successive frames at the first setting. A further seven data frames were recorded at a second orientation, in order to fill in blind-region data. Exposure times ranged between 22 and 32 h per frame. The observed diffraction peaks were indexed and matched to a wavelength range of 2.7–3.6 Å and to a d_{min} of 2.2 Å using the program *LAUEGEN* (Campbell, 1995). Integrated intensities were extracted using the $I/\sigma(I)$ method (Wilkinson *et al.*, 1988; Prince *et al.*, 1997) as implemented in the program *INTEGRATE*. Data from all images were scaled together and wavelength-normalized (to account for the spectral distribution) using the program *LAUENORM*. Data recorded at wavelengths less than 2.8 Å were in poor agreement with the remainder of the data, most likely owing to the fall-off in the spectral intensity distribution, and were excluded from wavelength-normalization calculations.

4. Results

Endothiapepsin crystals grow in two different crystal forms with the same monoclinic space group, $P2_1$. One of these forms has a significantly higher solvent content, a greater propensity for twinning and generally diffracts more weakly than the other form (Badasso *et al.*, 1992). Our co-crystals of the H261 complex belong to the second better ordered crystal form and have unit-cell parameters a = 43.0, b = 75.7, c = 42.9 Å, β = 97.0°.

The final data set for the H261 complex comprised 22 821 reflections with $I > 1.5\sigma(I)$, which reduced to 11 240 unique reflections with a merging R factor of 13.7%. The data are 80.4% complete to 2.2 Å resolution. A distinct advantage of the narrow (3.50 Å, dλ/λ = 20%) 'quasi-Laue' bandpass is that as the proportion of energy overlapped (harmonic) reflections is dependent on the ratio dλ/λ and is independent of the size of unit cell (Cruickshank *et al.*, 1987), almost all reflections (>99%) recorded on the LADI detector are singlets and amenable to standard analysis. Consequently, the low-resolution data are largely complete in the analyses reported here. Full statistics for processing of the highest resolution data set (H261) are given in Table 1.

5. Discussion

A major advantage of neutron diffraction is that H and/or D atoms, which cannot always be observed even at high resolution with X-ray analyses, are readily located in a neutron analysis because the scattering amplitudes of H/D for neutrons are closely similar to those of other biological atoms. A disadvantage is that the incoherent scattering cross-section of hydrogen for neutrons is large and diffraction spots appear against a substantial incoherent background that comes largely from the sample itself. Consequently, the signal-to-

noise ratio is unusually low. This can be compensated for in part by replacing exchangeable H atoms in the crystal with deuterium. As the neutron-scattering structure factors for hydrogen and deuterium differ in both amplitude and phase, neutron diffraction is then uniquely able to distinguish between hydrogen (negative Fermi density) and deuterium (positive Fermi density) positions in deuterated crystals. Analysis of the distribution and extent of H/D exchange in the crystal can then provide an elegant probe of group accessibility and mobility and of exchange dynamics.

We have demonstrated that high-resolution high-quality neutron diffraction data can be collected from crystals of the fungal aspartic proteinase endothiapepsin bound with an inhibitor. The primary objective of this analysis is to locate the positions of protons at the active site of an aspartic proteinase in complexes with transition-state analogues. This will provide much information needed to define the catalytic mechanism. Analysis of the pattern of H/D exchange in our endothiapepsin crystals will also provide insight into the flexibility of the molecule, which has been implicated in its catalytic function (Šali *et al.*, 1992). Delineation of the protonation states of buried carboxyl groups may help elucidate their proposed involvement in determining the pH optima of different members of the family. Refinement of the neutron structure of the complex reported above is in progress.

The unique information that is derived from neutron diffraction can be of exceptional importance in the understanding of biological mechanisms. The gains provided by the Laue neutron-diffraction (LADI) technique make this information more generally available, with the essential caveat that the sample size must still be of the order of 1 mm^3. For many protein systems, simply preparing larger crystals suitable for neutron diffraction analysis is likely to prove easier than preparing crystals that must necessarily diffract to beyond 1.2 Å in order to recover similar information in a cryo-crystallographic X-ray analysis.

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